Reference Gene Identification for Reverse Transcription-Quantitative Polymerase Chain Reaction Analysis in an Ischemic Wound-Healing Model

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Reference genes are often used in RT-quantitative PCR (qPCR) analysis to normalize gene expression levels to a gene that is expressed stably across study groups. They ultimately serve as a control in RT-qPCR analysis, producing more accurate interpretation of results. Whereas many reference genes have been used in various wound-healing studies, the most stable reference gene for ischemic wound-healing analysis has yet to be identified. The goal of this study was to determine systematically the most stable reference gene for studying gene expression in a rat ischemic wound-healing model using RT-qPCR. Twelve commonly used reference genes were analyzed using RT-qPCR and geNorm data analysis to determine stability across normal and ischemic skin tissue. It was ultimately determined that Ubiquitin C (*UBC*) and β-2 Microglobulin (*B2M*) are the most stably conserved reference genes across normal and ischemic skin tissue. *UBC* and *B2M* represent reliable reference genes for RT-qPCR studies in the rat ischemic wound model and are unaffected by sustained tissue ischemia. The geometric mean of these two stable genes provides an accurate normalization factor. These results provide insight on dependence of reference-gene stability on experimental parameters and the importance of such reference-gene investigations.

KEY WORDS: geNorm, stably conserved reference genes, chronic wounds, pre-clinical wound model

INTRODUCTION

Chronic ischemic wounds remain a major clinical challenge in long-term care, impacting patient quality of life, and place a significant burden on healthcare providers. The economic impact of chronic wound care has been estimated recently to be $6–15 billion/year.[1] The incidence of chronic wounds is likely to increase in coming years as the population ages, and rates of chronic disease, such as obesity, cardiovascular disease, and diabetes, continue to climb. These factors all contribute to chronic wound formation and are driving increased research in developing new, effective approaches to complement available techniques for chronic wound therapy.[2–4] Our understanding of the complexities of the normal wound-healing process has increased greatly with the growth of tissue engineering, as more sophisticated experimental techniques have become available[5,6]; the understanding of chronic wound pathobiology is less well-developed.[7] A systems approach to development of chronic wound therapies includes evaluation of gene expression to assess the impact of interventions on genes known to play a role in healing.

RT-quantitative PCR (qPCR) is a widely used technique to analyze gene expression through amplification of small quantities of RNA in representative tissue samples. To quantify RT-qPCR outcomes, the data should be normalized to a gene that is expressed ubiquitously across all samples. An appropriate reference gene is typically involved in cellular maintenance and works to regulate the basic functions of a cell. Incorporation of a reference gene with acceptably stable expression levels across tissue samples of interest provides a normalizing control to quantify RT-qPCR reactions. However, it is also well-established that a unique, universally stable reference gene is highly unlikely to occur in a specific model or tissue type.[8,9] It is therefore important to apply an investigative, methodological approach to identify appropriate reference genes to facilitate more accurate quantitative outcomes that can be compared across studies.

In the field of wound healing, Turabelidze et al.[10] have reported that the most appropriate reference genes in an acute wound murine model varied, not only from unwounded skin but also over the 5-day postwounding timeframe assessed. However, there is an absence of literature addressing the most stable reference gene for RT-qPCR

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analysis of ischemic wound healing. In preliminary work by our group (unpublished results), we have found that common reference genes are neither uniformly nor stably expressed across groups.

As noted above, it is important to determine the most stable and appropriate reference gene for use in models of ischemic wound healing to enhance the reliability and generalizability of RT-qPCR outcomes. The goal of our project was to identify the most stable reference gene for studying gene expression using RT-qPCR in a rat ischemic wound-healing model.

METHODS

Ischemic Wound Model and Tissue Samples

All animal procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (Cleveland, OH, USA). The rat ischemic wound model used in the current study[11] was based on that developed by Gould et al.[12] An aseptic surgical technique was used to minimize risk of postsurgical wound infections. Controlled ischemic wounds were created in 6-month-old male Fischer 344 rats by raising a 3 × 11.5-cm dorsal pedicle flap. The flap was longitudinally orientated, with the incision commencing rostrally at the T2 vertebra. Flap dimensions (26×100 mm) were drawn over the dorsum of the rat centered over the spine using a sterile surgical marker. Four, 6-mm wounds were created symmetrically about the midline, at a distance of 5.0 cm from the base of the scapula using a punch biopsy. Wounds were laterally separated such that two wounds were centered over the flap region and two over normal dermis, 1 cm lateral to the bipedical flap. The full-thickness punch wound included the skin and underlying panniculus carnosus but avoided the anterior muscle fascia. Biopsied tissue was removed by dissection in a plane between the panniculus carnosus and fascia. A dorsal, bipedicle flap was raised deep to the panniculus carnosus. Precut, nonreinforced, sterilized medical-grade silicone sheeting, 10 mil (0.25 mm) thickness (Sil-Tec; Technical Products, Lawrenceville, GA, USA) was placed under the flap. Skin flap edges and the silicone sheet were sutured to adjacent skin edges using interrupted, nonabsorbable sutures.

Tissue ischemia was monitored noninvasively by measurement of transcutaneous oxygen tension (T_cPO_2) using the Radiometer TCM4 system (Radiometer America, Westlake, OH, USA). The sensor was placed distal to the wounds on the caudal flap for ischemic measurements and on the haunch for control measurements. T_cPO_2 measurements were recorded preoperatively and on Postoperative Days 1, 7, 14, 21, and 28. Baseline T_cPO_2 was 25.8 ± 7.23 mmHg on normal skin. Ischemic flap T_cPO_2 was decreased compared with control locations and remained low throughout the postoperative period.

Ten normal tissue samples were harvested from baseline control wound biopsies during the initial wound creation. Ten ischemic tissue samples were harvested from the ischemic flap, 28 days after wound creation. Tissue was harvested using a sterile, 6-mm biopsy punch and then slightly offset of center in the direction of the hair growth. The smaller portion was finely diced and placed in a microcentrifuge tube containing RNAlater (Invitrogen, Carlsbad, CA, USA) for at least 24 h but no more than 72 h. With the use of a fresh RNase/DNase-free pipette tip for each sample, the RNAlater was then removed and samples stored at −80°C prior to further analysis.

Real-Time PCR

All recovered tissue samples were homogenized with TRIzol reagent (Invitrogen), and RNA was isolated using the PureLink RNA Mini Kit (Catalog Number 12183018A; Invitrogen). Total RNA was then checked for purity, and the concentration was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). cDNA was then synthesized from the extracted RNA using the SuperScript First-Strand Synthesis System (Catalog Number 11904-018; Invitrogen) with SuperScript II, according to the manufacturer's standard protocol. Specifically, a RNA/primer mix was prepared in sterile 0.5-ml tubes by mixing 400 ng Total RNA with 2 μL 10 mM dNTP mix and 2 μL 0.5 μg/μL oligo$(dT)_{12–18}$ and then diluting to 20 μL with DEPC-treated water. The RNA/primer mix was incubated at 65°C for 5 min and then placed on ice for at least 1 min. A 2× reaction mix was created by adding (in the following order): 4 μL 10× RT Buffer, 8 μL 25 mM $MgCl_2$, 4 μL 0.1 M DTT, and 2 μL RNaseOUT (40 U/μL concentrate). The 2× reaction mix (18 μL) was added to each RNA/primer mix. The two mixes were then centrifuged briefly before incubation at 42°C for 2 min. SuperScript II RT (2 μL; 100 units) was added to each tube before a second incubation at 42°C for 50 min, followed by termination of the reactions by incubation at 70°C for 15 min. The tubes were then placed on ice for at least 1 min prior to centrifuging briefly to collect the reactions. RNaseH (1–2 μL) was added to each tube, followed by incubation at 37°C for 20 min. Tubes were then stored at −20°C before spectrophotometric quantification and PCR.

The cDNA samples were then diluted to a concentration of 5 ng/μL in RNase/DNase-free water. A mastermix was made for each primer, comprising 66.7% 2× power iQ SYBR Green supermix (Catalog Number 170-8882; Bio-Rad, Hercules, CA, USA), 6.7% primer, and 26.7% water by volume. PCR reaction plates (96-well) were loaded manually by plac-

ing 15 μL mastermix in each well, together with 5 μL cDNA (25 ng) to give a total reaction volume of 20 μL.

Amplification was performed using the StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Thermocycling parameters followed the geNorm amplification protocol, specifically, initial enzyme activation for 10 min at 95°C, followed by 50 cycles of denaturation (15 s at 95°C) and data collection (60 s at 60°C). Run data were collected for export using the PCR system software and analyzed further as described below.

Primers

Twelve potential reference genes were investigated in the current study (Table 1). Eleven out of 12 were obtained in a commercially available geNorm reference gene kit (Catalog Number ge-PP-SY-12; Primer Design, Southampton, UK), designed for this reference gene study. The company guarantees >90% efficiency for its products, with a level of detail of ~10 copies/reaction. Previous work by our group (unpublished results) and others[10] has found that *TBP* was a reliable reference gene for acute wound models. *TBP* was therefore designed separately using National Center for Biotechnology Information/Primer-BLAST and obtained independently for the current study.

All samples for each reference gene were run on the same plate, with duplicates for each data point and negative controls, following the geNorm recommended layout guideline.

Gel electrophoresis was run to ensure the quality of the PCR reaction and verify that the *TBP* primer was of the same quality as those obtained from the geNorm kit. Reaction products were also sequenced to confirm amplification target accuracy.

Data Analysis

geNormPLUS, a proprietary reference gene analysis software module in qbasePlus (Biogazelle, Zwijnaarde, Belgium) was used to determine the relative stability of a reference gene within the population of reference genes studied.[13] The program algorithm determines the average paired variation of a specific reference gene against all other candidate genes (M). A low M-value is indicative of a high degree of stability. Thus, the gene with the lowest M-value is considered the most stable. geNormPLUS first uses comparative threshold values obtained from RT-qPCR and normalizes these values to the sample with the highest expression. After determining the M-values for all candidate genes, the gene with the highest M-value (and greatest instability) is removed at each step. This process continues with new M-values calculated for each gene until only genes below a specified M-threshold remain. These genes have the lowest M-values relative to the other candidate genes and thus, are the most stable reference genes in the model being evaluated.

RESULTS

geNormPLUS analysis first cleaned the data by removing outlying samples from further analysis if they had no stable reference gene expression. This resulted in the exclusion of

TABLE 1

Candidate Reference Genes for RT-qPCR Analysis

Gene name	Gene symbol	Accession number	Product length	Antisense primer GC%	Antisense primer Tm	Sense primer GC%	Sense primer Tm
18S Ribosomal RNA	18S	NR_046237	93	52.2	59.4	37	59.4
Actin, β	ACTB	NM_031144	98	50	56.1	55	56.7
ATP synthase	ATP5b	NM_134364	93	52.4	56.3	47.6	55.9
β-2 Microglobulin	B2M	NM_012512	95	55.6	58	50	57.3
Calnexin	CANX	NM_172008	116	43.5	56.1	47.6	56.2
Cytochrome C-1	CYC1	NM_001130491	135	55	56	66.7	55.7
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_017008	118	70.6	56	47.6	56.2
Malate dehydrogenase 1	MDH1	NM_033235	133	55	56.6	37.5	56.1
Ribosomal protein L13	RPL13	NM_031101	117	52.6	56	61.1	56.1
Topoisomerase	TOP1	NM_022615	101	61.1	56.2	47.6	56.1
Ubiquitin C	UBC	NM_017314	78	42.9	55.8	45.5	56.1
Tyrosine 3-mono-oxygenase	YWHAZ	NM_013011	140	43.5	56.3	52.6	55.8
TATA-binding protein	TBP	NM_001004198.1	23	60.9	66.4	56.5	66.2

GC%, Guanine-cytosine percent; Tm, melting temperature.

one ischemic tissue sample from further analysis. The cleaned experimental design and run layout were determined by the analysis software to be "perfect", making the results scientifically accurate and reliable.

In 28-day ischemic rat-skin tissue, geNormPLUS analysis determined that six of the 12 tested reference genes achieved an M = 1.0 threshold of stability (Fig. 1A). The use of all six stable reference genes was recommended to

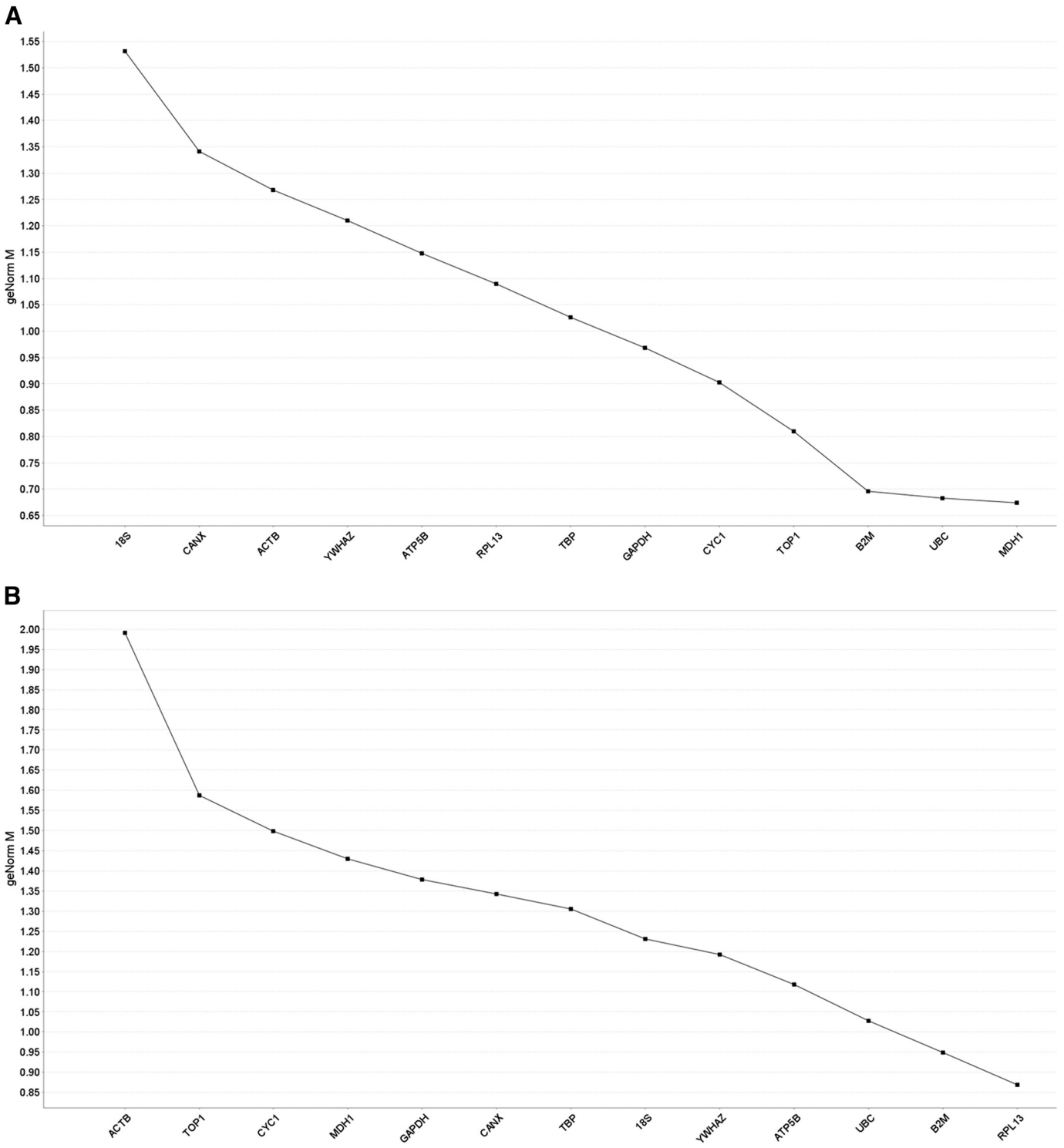

FIGURE 1

(*A*) geNorm analysis *M*-value stability of reference genes in ischemic tissue. (*B*) geNorm analysis *M*-value stability of reference genes in normal tissue.

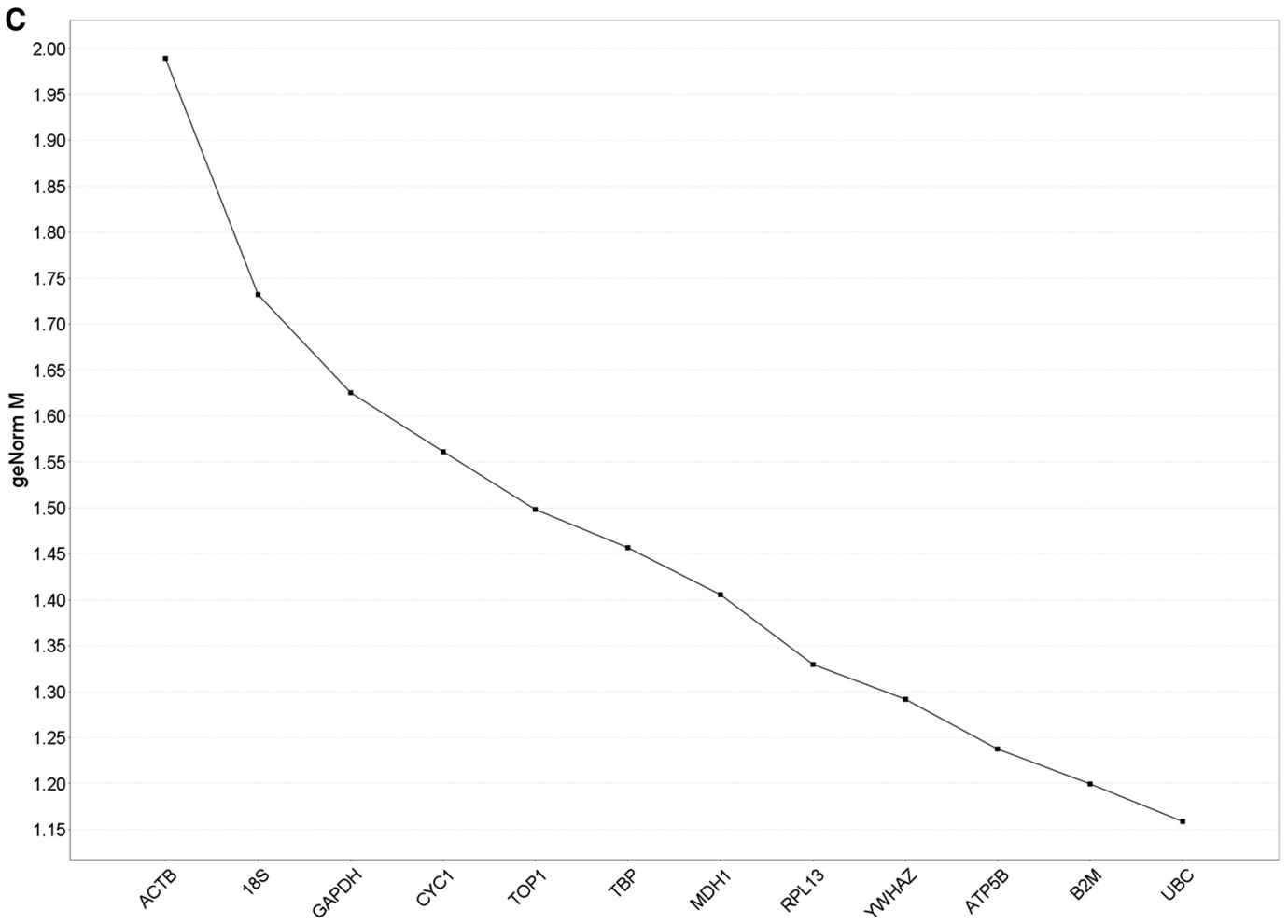

FIGURE 1—*Continued*

(*C*) geNorm analysis M-value stability of reference genes in ischemic and normal tissue concurrently.

achieve a geNorm V value <0.15 for ischemic tissue. The optimal normalization factor for ischemic rat-skin tissue can be calculated as the geometric mean of reference targets *GAPDH CYC1*, *TOP1*, *B2M*, *UBC*, and *MDH1*.

In normal rat-skin tissue, geNormPLUS analysis determined that only two of the 12 tested reference genes achieved an M = 1.0 threshold of stability (Fig. 1B). The use of eight reference genes was recommended to achieve a geNorm V value <0.15 for control tissue. The optimal normalization factor for normal rat skin can be calculated as the geometric mean of reference targets *CANX*, *TBP*, *18S*, *YWHAZ*, *ATP5b*, *UBC*, *B2M*, and *RPL13*.

Combining the two groups (Fig. 1C), geNormPLUS analysis determined that *UBC* and *B2M* were most stably conserved across all tissue samples.

DISCUSSION

Although the use of a reference gene is recommended for reliable and repeatable analysis of RT-qPCR, it is becoming increasingly clear that in most situations, a single reference gene is not sufficiently stable.[14] There is a paucity of literature on selection of reference genes for qPCR in skin. Brugè et al.[15] reported that whereas in vitro studies using human dermal fibroblasts frequently use qPCR, reference genes (often GAPDH or β-actin) generally appear to the selected without any validation of their expression stability. Under their experimental conditions, specifically UVA exposure, GAPDH and succinate dehydrogenase complex, subunit A (SDHA) were found to the be best reference genes. SDHA was also been reported by Balogh et al.[16] to be the best reference gene for studies of human keratinocytes exposed to UVB. In the field of wound healing, Turabelidze et al.[10] found that no single reference gene was temporally stable in an acute wound mouse model. The group found that *TBP*, *UBC*, and *CYC1* were the most stable reference genes for their model. Our geNormPLUS analysis of ischemic and normal rat skin indicated that *TBP* and *CYC1* had only moderate stability in ischemic tissue.

Reference genes above a threshold of M = 1.5 are

considered unstable and cannot provide reliable normalization. Interestingly, in our model, the normal skin tissue had less stable reference genes than the ischemic tissue. The least stable genes also varied, specifically *18S* in ischemic tissue and *ACTB* in normal tissue. When the ischemic and normal tissue was combined, it was found that these genes were the least stable overall. *18S* is a commonly used reference gene in RT-qPCR analysis and often assumed to be stable. This finding confirms that standard reference genes may not be appropriate for the model being studied.

It is important to verify the most stable reference gene for the specific model under investigation to obtain more accurate and reliable results in genes of interest. In our current study, the most stable genes recommended for use in determining the optimal normalization factor varied between the two groups. It can be an inefficient use of resources to obtain full optimization for each group when the goal is to determine changes between groups. It is therefore important to consider the overall study objectives and evaluate the most stable reference genes across all groups. In the current study, when the ischemic and normal tissue was combined, it was found that *B2M* and *UBC* were the most stable reference genes overall. It was also found that the stability of *RPL13* decreased markedly in ischemic tissue compared with normal skin. This reinforces the need to validate selection of reference genes under the interventional conditions of interest. Following the approach proposed by Vandesompele et al.,[17] it is recommended that the geometric mean of the two most stable reference genes, *UBC* and *B2M*, be used for normalization of RT-qPCR analysis in the rat ischemic wound model.

Conclusion

Our findings demonstrate that reference gene stability is dependent on many factors. Reference genes should be selected with care based on the specific model under investigation.

UBC and *B2M* represent reliable reference genes for RT-qPCR studies in the rat ischemic wound model, unaffected by sustained tissue ischemia. The geometric mean of these two stable genes provides an accurate normalization factor.

Our results provide a basis for the identification of genes differentially expressed in ischemic wounds, with the potential of discovering new markers of physiological responses to treatment interventions.

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DISCLOSURE

The authors have no conflicts of interest to report.

REFERENCES

1. Markova A, Mostow EN. US skin disease assessment: ulcer and wound care. *Dermatol Clin* 2012;30:107–111.
2. Gist S, Tio-Matos I, Falzgraf S, Cameron S, Beebe M. Wound care in the geriatric client. *Clin Interv Aging* 2009;4:269–287.
3. Wong VW, Gurtner GC. Tissue engineering for the management of chronic wounds: current concepts and future perspectives. *Exp Dermatol* 2012;21:729–734.
4. Game FL, Hinchliffe RJ, Apelqvist J, et al. A systematic review of interventions to enhance the healing of chronic ulcers of the foot in diabetes. *Diabetes Metab Res Rev* 2012;28(Suppl 1):119–141.
5. Raman D, Sobolik-Delmaire T, Richmond A.Chemokines in health and disease. *Exp Cell Res* 2011;317:575–589.
6. Rafehi H, El-Osta A, Karagiannis TC. Genetic and epigenetic events in diabetic wound healing. *Int Wound J* 2011;8:12–21.
7. Tomic-Canc M, Agren MS, Alvararez OM. Epidermal repair and the chronic wound. In Rovee DT, Maibach HI (eds): *The Epidermics in Wound Healing*. Baton Rouge, LA, USA: CRC, 2004;25–57.
8. Ross DT, Scherf U, Eisen MB, et al. Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* 2000; 24:227–235.
9. Warrington JA, Nair A, Mahadevappa M., Tsyganskaya M. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiol Genomics* 2000;2:143–147.
10. Turabelidze A, Guo S, DiPietro LA. Importance of housekeeping gene selection for accurate reverse transcription-quantitative polymerase chain reaction in a wound healing model. *Wound Repair Regen* 2010;18:460–466.
11. Henzel MK, Howe DS, Graebert J, Bogie KM. Optimization of a rat ischemic wound model for evaluation of the effects of electrotherapy on wound healing. 22nd Annual Meeting of the Wound Healing Society, Symposium on Advanced Wound Care–Spring/Wound Healing Society Joint Meeting. *Wound Rep Regen*. Oxford, UK: Blackwell Publishing, 2012.
12. Gould LJ, Leong M, Sonstein J, Wilson S. Optimization and validation of an ischemic wound model. *Wound Repair Regen* 2005;13:576–582.
13. McCulloch RS, Ashwell MS, O'Nan AT, Mente PL. Identification of stable normalization genes for quantitative real-time PCR in porcine articular cartilage. *J Anim Sci Biotechnol* 2012; 3:36.
14. De Jonge HJ, Fehrmann RS, de Bont ES, et al. Evidence based selection of housekeeping genes. *PLoS One* 2007;2:e898.
15. Brugè F, Venditti E, Tiano L, Littarru GP, Damiani E. Reference gene validation for qPCR on normoxia- and hypoxia-cultured human dermal fibroblasts exposed to UVA: is β-actin a reliable normalizer for photoaging studies? *J Biotechnol* 2011; 156:153–162.
16. Balogh A, Paragh G Jr, Juhász A, et al. Reference genes for quantitative real time PCR in UVB irradiated keratinocytes. *J Photochem Photobiol B* 2008;93:133–139.
17. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3:RESEARCH0034.